Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Evaluation by the Relative Potency Method of *In Vitro* Enzyme Immunoassays Used in Testing of Veterinary Vaccines

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Supplemental Assay Method for Evaluation by the Relative Potency Method of *In Vitro* Enzyme Immunoassays Used in Testing of Veterinary Vaccines

1. Introduction

This calculation method is used for evaluation of *in vitro* enzyme immunoassays (EIA or ELISA) in serial release testing of veterinary vaccines. The method compares a reference vaccine (reference) and the serial being tested (serial) by a parallel line, relative potency (RP) method. The reference shall be a serial of product produced per a filed Outline of Production or a purified reference consisting of only the agent, a subunit of the agent, or a nonadjuvanted harvested culture of microorganisms. The reference shall be an Animal and Plant Health Inspection Service (APHIS) approved reference that has been directly or indirectly correlated to a host animal efficacy trial in which sufficient protection was elicited to pass the minimum requirements set by APHIS for the agent being tested. The test methods vary, but each shall be an *in vitro* antigen quantitation method approved by APHIS.

The evaluation determines regression segments for all combinations of 3 or more consecutive dilutions for both the reference and the serial. The optical density (OD) (inverse $\log_{10}$ of transmittance), without transformation, and the natural log ($\log_e$, approximating $\log_2$) of the dilution are used in the calculations. A correlation coefficient, r, is calculated for each line to measure the goodness of fit. Each line is evaluated by a t-test to determine if the slope is significantly different from 0. Lines not meeting minimum criteria for goodness of fit and slope are considered invalid and are not used in further calculations. All possible combinations between valid regression lines of the reference and the serial are compared for parallelism. An average slope is calculated for combinations of lines that are considered to be parallel.

A weighted scoring system awards regression lines that have a high correlation coefficient and a high t-test value. Combinations of valid lines are also rewarded for steepness of slope and slope ratios closest to 1. The relative potency values of the 3 highest total scores are used in determining if a serial has a greater antigen content than the reference.

2. Materials

2.1 Equipment/instrumentation

2.1.1 *In vitro* enzyme immunoassay test results

2.1.2 Computer software program (optional)

A computer software program utilizing the parameters detailed in this Supplemental Assay Method is available from the Center for Veterinary Biologics-Laboratory (CVB-L). Version 3.1 of the software is written in the

QuickBASIC programming language for an IBM or IBM-compatible computer using the DOS operating system. Version 4.0 is written in the VisualBASIC programming language for use with Windows 95, 98, ME, NT, or XP operating systems. The software is supplied as a compiled, executable program. Appendix A may be used to request the software. Please limit the number of copies requested from the CVB-L to 1 copy per company site. Additional copies of the program can be made for use and distribution within the company. Please refer any request for the software from outside of your firm to the CVB-L. This will help the CVB-L to maintain a list of users of the program in the event additional modifications or upgrades need to be distributed.

2.1.3 Instrumentation for calculation

2.1.3.1 A hand-held calculator may be used when manually calculating.

2.1.3.2 When using the optional computer software with an IBM or IBM-compatible computer the following minimum requirements are required:

1. Version 3.1

   Version 3.1 or higher of the Microsoft disk operating system (DOS).

   A 3.5" floppy drive able to read 1.44K disks.

   A monochrome, CGA, EGA, or VGA video system.

2. Version 4.0

   Windows 95 operating system

   Computer with 166 MHZ and 16 MB RAM

   CD-ROM drive

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall be familiar with enzyme-linked immunoassays. If using the optional software, a minimal knowledge of personal computers and data entry is required.

3.2 Preparation of equipment/instrumentation-computer calculations (optional)

3.2.1 The optional program allows from 3 to 11 consecutive dilutions and up to 10 replicates of each dilution for both the reference and the serial. A reference and up to 5 serials can be compared. The option to remove outliers is available. The program allows entry of a minimum OD and a minimum slope value. Values less than these minimums are not used in calculations. Provision is made for identity of reference, serial, testing information, and date. The results are printed with these identifiers. A graphic representation of the reference versus the serial is available for viewing or printing.

4. Performance of the test - calculation method

4.1 Lines from all possible combinations containing 3 or more consecutive dilutions for both the reference and the serial are evaluated by a simple linear regression of Y on X:

$$Y_i = \alpha + \beta X_i$$

where:

α = population parameter for the Y intercept

β = population parameter for the regression coefficient (slope)

X_i = $\log_e$ of the dilution

= natural log of the dilution

= ln(dilution)

Y_i = OD for dilution, X_i

If a minimum OD value greater than 0.05 has been established for the test assay, then all OD values less than the minimum should be considered invalid and not used in further calculations. Otherwise all values less than 0.05 are considered invalid.

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The best approximation of β is obtained by b:

$$b = \frac{SS_{xy}}{SS_{xx}}$$

where:

b = slope of the regression line

SS_{xy} = sum of squares for the cross product

$$= \sum_{i=1}^{n} x_i y_i = \sum_{i=1}^{n} (X_i - \overline{X})(Y_i - \overline{Y})$$

SS_{xx} = sum of squares for X

$$= \sum_{i=1}^{n} x_i^2 = \sum_{i=1}^{n} (X_i - \overline{X})^2$$

n = number of dilutions

$x_i = X_i - \overline{X}$

$y_i = Y_i - \overline{Y}$

$x_i y_i = (X_i - \overline{X})(Y_i - \overline{Y})$

$\overline{X}$ = mean of the observed dilutions

$$= \frac{\sum_{i=1}^{n} X_i}{n}$$

$\overline{Y}$ = mean of the observed OD

$$= \frac{\sum_{i=1}^{n} Y_i}{n}$$

If a minimum slope value has been established for the test assay, then all lines with slope values less than the minimum are considered invalid and are not used in further calculations.

The best approximation of α is obtained by *a*, which can be derived by substitution:

$$a = \overline{Y} - b\overline{X}$$

where:

a = *Y* intercept

4.2 The sign of the slope of the line characteristic for the type of assay being conducted is determined. For indirect EIA, capture EIA, etc., the slope is expected to be negative (*i.e.*, with increasing dilution [decreasing antigen] the OD will decrease). For a competitive EIA, the slope is expected to be positive (*i.e.*, a greater OD will occur with increasing dilution). All lines with signs other than expected are considered invalid and are not used in further calculations.

4.3 When 3 or more replicates of each dilution are conducted, data points that exceed plus or minus 2 standard deviations (±2 SD) from the mean can be removed as outliers:

$\overline{Y_{i\cdot}}$ = sample mean of Y for a dilution

$SD_{Y_{i\cdot}}$ = standard deviation of Y for a dilution

$$= \sqrt{\frac{\sum_{j=1}^{n_r} Y_{ij}^2 - \frac{(\sum_{j=1}^{n_r} Y_{ij})^2}{n_r}}{n_r - 1}}$$

where:

n_r = number of replicates for a dilution

Y_{ij} = the OD for each replicate for a dilution

outliers = any replicate outside of the range $\overline{Y_{i\cdot}} \pm 2\ SD_{Y_{i\cdot}}$

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Outlier removal is optional, but if any outliers are removed, then all outliers meeting the criteria shall be removed for all dilutions. If more than 40% of the replicates for a dilution are removed as outliers, then that dilution shall not be used in further calculations.

4.4 The correlation coefficient is determined for each line:

$$r^2 = \frac{SS_R}{SS_{yy}}$$

$$r = \sqrt{r^2}$$

where:

r^2 = coefficient of determination

r = correlation coefficient

SS_R = sum of squares from the regression

$$= \frac{(SS_{xy})^2}{SS_{xx}}$$

SS_{yy} = sum of squares for Y

$$= \sum_{i=1}^{n} Y_i^2 = \sum_{i=1}^{n} (Y_i - \overline{Y})^2$$

Lines with an r value less than 0.95 ($r < 0.95$) are considered invalid and are not used in further calculations.

4.5 Each line is evaluated for having a slope significantly different from 0:

$$t = \sqrt{\frac{SS_R}{\left(\frac{SS_E}{(n-2)}\right)}}$$

where:

t = t-value

SS_E = sum of squares for lack of fit (*experimental error*)

= $SS_{yy} - SS_R$

The t-value is compared to a value obtained from a standard t table. The t-value for comparison is determined at the 5% significance level with one-tail and n-2 degrees of freedom ($t_{0.05,(n-2)}$). All lines with nonsignificant t-test values are considered statistically equal to 0 and invalid. Those lines are not used in further calculations.

4.6 All valid lines are evaluated for an r-value greater than or equal to 0.98 ($r \geq 0.98$) and for a t-value at the 1% significance level with one-tail and n-2 degrees of freedom ($t_{0.01,(n-2)}$). A score is then assigned:

Significance level for t-test	r-value	Score
>0.01,≤0.05	≥0.95,<0.98	0
>0.01,≤0.05	≥0.98	2.5
≤0.01	≥0.95,<0.98	2.5
≤0.01	≥0.98	5

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4.7 The ratio of the slopes for all possible combinations of 1 valid line from the reference and 1 valid line from the serial are compared by:

$$\text{slope ratio} = \frac{\text{smaller slope}}{\text{larger slope}} = \frac{b_R}{b_S} \text{ or } \frac{b_S}{b_R}$$

where:

b_R = slope of the line for the reference

b_S = slope of the line for the serial

Combinations having slope ratios less than 0.80 (<0.80) are considered nonparallel and are not used in further calculations.

4.8 Each valid combination of 1 line from the reference and 1 line from the serial is assigned a score based on the closeness of the combination's slope ratio to 1:

Slope Ratio	Score
≥0.98	10
≥0.96, <.98	9
≥0.94, <.96	8
≥0.92, <.94	7
≥0.90, <.92	6
≥0.88, <.90	5
≥0.86, <.88	4
≥0.84, <.86	3
≥0.82, <.84	2
≥0.80, <.82	1

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4.9 For each valid combination of 1 line from the reference and 1 line from the serial, a common, or average, slope is determined by:

$$b_c = \frac{SS_{xyR} + SS_{xyS}}{SS_{xxR} + SS_{xxS}}$$

where:

b_c = common slope

SS_{xyR} = sum of squares of cross products for the reference

SS_{xyS} = sum of squares of cross products for the serial

SS_{xxR} = sum of squares of X for the reference

SS_{xxS} = sum of squares of X for the serial

Valid combinations are ranked by the absolute value of the common slope (absolute value of $b_c = |b_c|$) from highest to lowest. The top 10 combinations are assigned scores from 20 to 2 based on their rank, with the combination having the steepest slope being assigned a score of 20 and each subsequent combination with less slope being assigned a score according to twice its rank (*i.e.*, 2 times the rank = 18, 16, 14...). In cases where 2 or more slopes within the 10 steepest slopes are equal, a score of twice the average of the ranks is assigned to each of the combinations involved. A score of 0 is assigned to slopes not within the top 10 steepest slopes.

4.10 For each valid combination of 1 line from the reference and 1 line from the serial, an RP is calculated by:

$$\log_e(RP) = (\overline{X_S} - \overline{X_R}) - \left(\frac{\overline{Y_S} - \overline{Y_R}}{b_c} \right) \text{ for negative slope assays}$$

$$RP = \text{antilog } [\log_e(RP)]$$

where:

$\log_e(RP)$ = natural log of the RP

RP = relative potency

$\overline{X_S}$ = mean of X for the serial

$\overline{X_R}$ = mean of X for the reference

$\overline{Y_S}$ = mean of Y for the serial

$\overline{Y_R}$ = mean of Y for the reference

5. Interpretation of the test results

5.1 All scores for each valid combination consisting of 1 line from the reference and 1 line from the serial are totaled. The scores will range from 0 to 40. The relative potencies for the top 3 total scores (or all RP values if there are less than 3 valid combinations) are used to determine if a serial has greater antigen content than the reference. If valid line combinations with the same total score occur within the top 3 scores, the RP value of all lines with the same score are reported. For a serial to be satisfactory, any 1 of these top scores must have an RP greater than or equal to the required minimum contained in a filed APHIS Outline of Production or special outline for the product being tested. The highest RP of the top scores shall be considered the RP value for reporting purposes.

5.2 Testing criteria

5.2.1 Testing criteria shall be per the Code of Federal Regulations (9 CFR 113.8.c). A test that results in no valid lines is considered a "no test" and may be repeated. An initial test that results in valid lines that are not parallel is considered a valid equivocal test. Release of the serial may not be based on such test since the result cannot be termed "satisfactory" or "unsatisfactory."

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Test results and interpretation:

	Test 1	Test 2 (retest 1)	Test 3 (retest 2)	Test 4 (retest 3)	Result	9 CFR Reference
1	≥RP				SAT	113.8.c.3
2	<RP	≥RP	≥RP		SAT	113.8.c.5.i
3	<RP	≥RP	EQUIV	≥RP	SAT	113.8.c.5.ii
4	<RP	≥RP	EQUIV	EQUIV	NER	113.8.c.5.ii
5	<RP	≥RP	<RP		UNSAT	113.8.c.5.i
6	<RP	EQUIV	<RP		UNSAT	113.8.c.5.i
7	<RP	EQUIV	≥RP	≥RP	SAT	113.8.c.5.ii
8	<RP	EQUIV	≥RP	EQUIV	NER	113.8.c.5.ii
9	<RP	EQUIV	EQUIV		NER	113.8.c.5.ii
10	<RP	<RP			UNSAT	113.8.c.5.i
11	EQUIV	≥RP			SAT	113.8.c.4.i
12	EQUIV	EQUIV	≥RP		SAT	113.8.c.4.i
13	EQUIV	EQUIV	<RP		NER	113.8.c.4.i
14	EQUIV	EQUIV	EQUIV	≥RP	SAT	113.8.c.4.i
15	EQUIV	EQUIV	EQUIV	<RP	NER	113.8.c.4.i
16	EQUIV	EQUIV	EQUIV	EQUIV	NER	113.8.c.4.ii
17	EQUIV	<RP	≥RP	≥RP	SAT	113.8.c.4.ii
18	EQUIV	<RP	≥RP	<RP	UNSAT	113.8.c.4.ii
19	EQUIV	<RP	≥RP	EQUIV	NER	113.8.c.4.ii
20	EQUIV	<RP	EQUIV		NER	113.8.c.4.ii
21	EQUIV	<RP	<RP		UNSAT	113.8.c.4.ii

RP = relative potency

EQUIV = equivocal test

SAT = satisfactory test

UNSAT = unsatisfactory test

NER = not eligible for release (see provision in 9 CFR, Part 113.8 for evaluation of test protocol and approval for retesting)

6. Report of test results

Test results are reported as "satisfactory," "unsatisfactory," "no test," or "not eligible for release."

7. References

7.1 Finney, D.J. Quantitative dose-response relations. *Statistical Method in Biological Assay*, 3rd ed. London: Charles Griffin & Co, 1978:39-68.

7.2 Neave H.R. *Statistics tables for mathematicians, engineers, economists and the behavioral and management sciences.* Boston: George Allen & Unwin, 1978. (Several similar references containing statistical tables are available and are equivalent.)

7.3 Finney, D.J. Parallel line assays. *Statistical Method in Biological Assay*, 3rd ed. London: Charles Griffin & Co, 1978:69-104.

8. Summary of revisions

8.1 MVSAM0318.01. Replaced draft SAM 318, dated July 17, 1992. The document was rewritten to meet the current NVSL/CVB QA requirements. No significant changes were made from the previous method.

8.2 MVSAM0318.02. Replaced SAM 318.01, dated March 19, 1998.

8.2.1 Appendix A was revised to reflect the new computer media distribution options with the release of version 3.1 of the U. S. Department of Agriculture Veterinary Biologics Program's Relative Potency Calculation Software (*RelPot*).

8.2.2 The calculation method for the relative potency in **Section 4.10** was corrected for positive slope assays. Previously the calculation method for positive slope assays was the inverse of the correct method.

8.3 MVSAM0318.03

8.3.1 Revised to incorporate *RelPot* version 4.0 in Section 3.

8.3.2 Revised Appendix A to allow selection of *RelPot* version.

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Appendix A
SAM - 318

TO: Director's Office
Center for Veterinary Biologics-Laboratory
P.O. Box 844
Ames, IA 50010

Please send a copy of the U.S. Department of Agriculture, Veterinary Biologics Program's *Relative Potency* Calculation Software to:

Firm: _____

Name: _____

Address: _____

_____

_____

Person using the program and designated to receive upgrades if different from above:

Name: _____

Address: _____

_____

_____

Version and disk size desired:

☐ Version 3.1 (DOS version)

☐ 3.5" floppy disk

☐ Compact disk (CD-ROM)

☐ Version 4.0 (Windows version), supplied on CD-ROM